SMB

A Biologically Active Protein - Collagen Fragment HF-COLL-18/514cf - for Inhibiting Tumor Growth and Capillary Proliferations

The present invention relates to a peptide (protein) which is capable of affecting the growth of cells. Collagen fragment HF-COLL-18/514cf and fragments and/or derivatives thereof as well as a medicament containing the natural and synthetic peptides can be employed for diagnostic or therapeutic purposes.

The invention relates to a process for obtaining a protein in a pure or partially purified form from human body fluids which protein is capable of affecting the growth of cells in an astonishing way, thereby inhibiting vascular and tumor growth. A similar substance has recently been detected in mice (O'Reilly et al., 1997, Cell, Vol. 88, page 277). The present substance, in contrast, is characterized in that it can be recovered, in particular, from hemofiltrate or hemodialyzate filtered from human blood. The substance has been designated HF-COLL-18/514cf and may be used for (1) analyzing diseases and (2) as a medicament.

The substance HF-COLL-18/514cf was first obtained from the hemofiltrate of patients suffering from renal diseases after ultrafiltration with a hemodialysis device, and characterized in terms of its molecular mass and the 60 N-terminal amino acids. For the preparation of HF-COLL-18/514cf, a patented process (Forssmann, 1988; Offenlegungsschrift DE 36 33 707 A1) has been sophisticated which had been invented for recovering proteins from hemofiltrate. Among the molecules obtained by this process having a molecular weight of below 20 kilodalton which are

filtered off in veno-venous or arterio-venous shunting, the fractions containing the HF-COLL-18/514cf can surprisingly be recognized by mass spectrometry. It has further been found in other specialized processes that this substance could astonishingly be purified until a homogeneous protein was finally identified and its structure elucidated. Surprisingly, this substance is the fragment of a protein which to date has only been known on the cDNA level (Oh et al., 1994, Genomics, Vol. 19, page 494). The value of this invention in characterized in that this substance can be purified from hemofiltrate, which had been considered worthless, to be used as an economically utilizable substance.

Thus, a compound has been isolated the structure of which had been unknown and the site of formation of which in the body is still unclear. The therapeutic and economic use has been tested, and HF-COLL-18/514cf has surprisingly been recognized as an important circulating peptide of human blood.

The substance mentioned, HF-COLL-18/514cf, can be obtained by chemical synthesis and by genetic engineering and may be used for numerous other purposes, inter alia, for analysis in human blood as a pathognomonic diagnostic feature of diseases of vascular growth, of tumor growth, and of metastases.

Thus, the present invention relates to a novel peptide, HF-COLL-18/514cf, its preparation, medicaments containing it as well as formulations containing it and its use for preparing them, as well as its natural and pharmacologically compatible derivatives, especially amidated, acetylated, phosphorylated and glycosylated HF-COLL-18/514cf derivatives and fragments of this peptide. An average molecular weight of 18494 u dalton could be determined by mass spectrometry.

The blood peptide HF-COLL-18/514cf has the following amino acid sequence:

Val-Ala-Leu-Asn-Ser-Pro-Leu-Ser-Gly-Gly-Met-Arg-Gly-Ile-Arg-Gly-Ala-Asp-Phe-Gln-Cys-Phe-Gln-Gln-Ala-Arg-Ala-Val-Gly-Leu-Ala-Gly-Thr-Phe-Arg-Ala-Phe-Leu-Ser-Ser-Arg-Leu-Gln-Asp-Leu-Tyr-Ser-Ile-Val-Arg-Arg-Ala-Asp-Arg-Ala-Ala-Val-Pro-Ile-Val-Asn-Leu-Lys-Asp-Glu-Leu-Leu-Phe-Pro-Ser-Trp-Glu-Ala-Leu-Phe-Ser-Gly-Ser-Glu-Gly-Pro-Leu-Lys-Pro-Gly-Ala-Arg-Ile-Phe-Ser-Phe-Asp-Gly-Lys-Asp-Val-Leu-Arg-His-Pro-Thr-Trp-Pro-Gln-Lys-Ser-Val-Trp-His-Gly-Ser-Asp-Pro-Asn-Gly-Arg-Arg-Leu-Thr-Glu-Ser-Tyr-Cys-Glu-Thr-Trp-Arg-Thr-Glu-Ala-Pro-Ser-Ala-Thr-Gly-Gln-Ala-Ser-Ser-Leu-Leu-Gly-Gly-Arg-Leu-Leu-Gly-Gln-Ser-Ala-Ala-Ser-Cys-His-His-Ala-Tyr-Ile-Val-Leu-Cys-Ile-Glu-Asn-Ser-Phe-Met-Thr-Ala-Ser

The peptide HF-COLL-18/514cf provided by the present invention is now a readily available drug with the biological and therapeutic activity of a natural analogue of the substance occurring in blood.

The present invention provides a production process for said HF-COLL-18/514cf as well as the use of HF-COLL-18/514cf as a medicament for various therapeutic and diagnostic indications. HF-COLL-18/514cf may be used as a high-purity material, or in a partially purified mixture of peptides if this is sufficient for the particular use.

The peptide according to the invention, its derivatives and fragments can be prepared by various processes, e.g., through prokaryotic or eukaryotic expression and optionally chromatographic purification. It can further be isolated from human blood, e.g., by per se known chromatographic methods. Finally, HF-COLL-18/514cf or its derivatives or fragments can be prepared from the amino acids contained in the stated sequence in protected form by common methods of solid-phase and liquid-phase synthesis. After deprotecting, it can be purified by common chromatographical methods.

The medicinal formulation according to the invention contains HF-COLL-18/514cf or a physiologically compatible salt of HF-COLL-18/514cf. The form and composition of the medicament which contains the HF-COLL-18/514cf depends on the route of administration. Human HF-COLL-18/514cf can be administered parenterally, intranasally, orally, intravenously, intramuscularly, intracutaneously, intrathecally, locally-topically or transpulmonarily. Preferably, HF-COLL-18/514cf is manufactured into an injection preparation, either as a solution or as a lyophilizate to be dissolved immediately prior to use. The medicinal formulation may additionally contain additives which are required by the filling technique, contribute to solubility, stability or sterility of the medicament, or increase the efficiency of intake into the body. It is particularly advantageous to use the lyophilizated form taken up with mannite in sterile ampoules to be dissolved in physiological saline and/or infusions for repeated individual injection and/or permanent infusion in amounts of 30 μg to 30 mg of pure HF-COLL-18/514cf per unit dose.

The daily dose of HF-COLL-18/514cf to be administered depends on the indication and on the particular derivatives used. With i.v./i.m. injection, it is in the range of from 100 to 1200 units (μg)/day, and with daily subcutaneous injection, it preferably ranges from 300 to 2400 units (μg)/day.

The peptide HF-COLL-18/514cf according to the invention is characterized in that it is particularly suitable for long-term therapy of tumor diseases or other diseases which are characterized by uncontrolled vascular growth, and that it does not trigger an immune response in permanent treatment. The preparation according to the invention is particularly suitable for a combination therapy involving chemotherapy and radiotherapy, or subsequent to chemotherapy or radiotherapy in cancer.

The preparation according to the invention can further be employed as an agent for therapy and diagnosis in vascular diseases of the supporting and connective tissue, the respiratory tract,

the cardiovascular system and the urogenital system, the nervous system and the eyes since it can be used for the preparation of human-compatible antibodies which are suitable for detecting and affecting changes of vascular growth in these organs.

Such antibodies are basically obtainable by immunizing animals with the peptide according to the invention and/or its fragments, or by using hybridoma technology.

The present invention also relates to a process for the treatment of patients in need of HF-COLL-18/514cf or its derivatives or fragments by the administration of therapeutic amounts of HF-COLL-18/514cf. Patients suffering from overproduction of HF-COLL-18/514cf or its derivatives or fragments require the administration of therapeutic amounts of an antagonist/inhibitor of HF-COLL-18/514cf.

The medicament according to the invention is suitable for the treatment of diseases of the human organism, especially in connection with capillary proliferations, carcinoses, diseases involving the cardiovascular and nervous systems, diseases involving the intugement and the sense organs, especially the eyes.

According to the invention, there is claimed the use of the peptide or its derivatives, the fragments or the antibody according to the invention for the preparation of a medicament for the treatment of disorders in inflammatory processes, disturbed inflammatory reactions, proliferation and maturation disorders of the blood-forming system, of systemic diseases in an overproduction or deficiency of HF-COLL-18/514cf, especially when, e.g., antibodies have been formed against it in former applications, or the use of HF-COLL-18/514cf in substitution therapy, chronic diseases, partially accompanied by the diseases mentioned by using it in a suitable form for the treatment due to electrolytic activity in tumor and vascular diseases.

The medicament according to the invention is suitable for the treatment of acute diseases of the kinds mentioned above by using it in a suitable form for the treatment of these diseases in intensive care.

A further use of the peptide according to the invention, its fragments or the antibody according to the invention is for the diagnosis of diseases by preparing specific antibodies against synthetic fragments or the whole peptide or its derivatives and fragments and, e.g., measuring the blood concentration of HF-COLL-18/514cf by immunoassays.

Thus, a diagnostic agent containing the peptide according to the invention, its fragments or antibodies according to the invention for test systems for checking the levels of this substance in tissues, plasma, urine and cerebrospinal liquor is also a subject matter of the invention. The diagnostic agent according to the invention is particularly suitable as a marker for certain carcinoses and for functional disorders of blood vessels, bone marrow, lymph organs, the gastro-intestinal tract, the immune system and for inflammatory and neoplastic processes.

The invention will be further explained by means of the following Examples.

Example 1: Isolation and Characterization of Circulating HF-COLL-18/514cf from Human Hemofiltrate

As the starting material, there was used hemofiltrate which is obtained in large amounts in the treatment of renal insufficiency patients and contains all plasma components up to a molecular size of about 20,000 dalton.

I. Recovery of the raw peptide material

The hemofiltrate was obtained using a Sartorius hemofiltration plant and cellulose triacetate filters with an exclusion size of

20,000 dalton (SM 40042, Sartorius, Göttingen, Germany). The filtrate was derived from renal insufficiency patients which were in a stable metabolic condition from long-term hemofiltration, and protected from proteolytic degradation immediately after recovery by acidification and cooling at 4°C. In four extractions with a cation exchange column (TSK SP 650(M), Merck, Darmstadt, Germany), 2860 l of hemofiltrate was processed. 93% of the pooled extracts were successively eluted from the above-mentioned column material by different buffers having different pH values. The raw fractions were subsequently subjected to freeze-drying.

II. Preparative RP chromatography

500 mg out of 2200 mg of the last raw fraction was roughly separated by hydrophobicity by means of preparative RP chromatography. Fractions were collected from a PrepPak Cartridge with dimensions of 47 x 300 mm supplied by Waters. Fraction 31 was used for further purification.

Device:	BioCad HPLC (Perseptive Biosystems, Freiburg, Germany)
Column:	Waters PrepPak Cartridge 47 x 300 mm
Material:	Vydac, 300 Å, 15 - 20 µm
Eluent A:	water with 10 mM HCl
Eluent B:	methanol with 10 mM HCl
Gradient:	0 - 50% eluent B 28.57 min
	50 - 95% eluent B 61.43 min
	95% eluent B 5.71 min
Flow rate:	35 ml/min
Fractions:	50 ml or 1.43 min
Detection:	230 nm and 280 nm

III. First analytical RP HPLC

Ultraviolet absorption during analytical RP chromatography of fraction 31 which had been obtained from the separation in figure 1. In a gradient on a Vydac column (10 x 250 mm, steel coat,

material: RP C18, 300 Å, 5 µm), a further separation could be achieved. The eluents were water with 0.1% by volume of trifluoroacetic acid, and acetonitrile with 0.1% by volume of trifluoroacetic acid.

Device:	Kontron HPLC plant
Column:	Vydac, steel coat, 10 x 250 mm
Material:	Vydac RP-C18, 300 Å, 5 µm
Eluent A:	water with 0.1% by volume of trifluoroacetic acid
Eluent B:	acetonitrile with 0.1% by volume of trifluoroacetic acid
Gradient:	0 - 60% eluent B 50 min
	60 - 80% eluent B 5 min
	80 - 0% eluent B 5 min
Flow rate:	2 ml/min
Fractions:	2 ml or 1 min
Detection:	230 nm

IV. Detection of the molecular mass of HF-COLL-18/514cf by means of MALDI TOF mass spectrometry

With a MALDI mass spectrometer RBT II (Vestec/PerSeptive, Houston, Texas, USA), mass spectra of the purified native HF-COLL-18/514cf from the preparation in step III were measured using α-cyano-4-hydroxycinnamic acid as the matrix. In fractions 45 and 46, peaks of the singly, doubly and triply protonated molecule could be seen with a molecular mass of about 18500 u. In addition, various minor components could be seen.

V. Second analytical RP-HPLC

In a final analytical RP chromatography of pooled fractions 45 and 46 which had been obtained from the separation in step III, highly purified HF-COLL-18/514cf could be isolated in fraction 25.

Device:	Kontron HPLC (Kontron, Munich, Germany)
Column:	YMC, steel coat, 4.6 x 250 mm
Material:	YMC RP-C18, 300 Å, 5 µm
Eluent A:	water with 0.1% by volume of trifluoroacetic acid
Eluent B:	80% acetonitrile, 20% water (v/v) with 0.1% by volume of trifluoroacetic acid
Gradient:	0 - 30% eluent B 5 min
	30 - 80% eluent B 150 min
	80 - 100% eluent B 5 min
	100 eluent B 5 min
Flow rate:	0.6 ml/min
Fractions:	manually collected
Detection:	230 nm and 280 nm

VI. Determination of purity by capillary zone electrophoresis

5 µl of fraction 25 was directly used for measuring in capillary zone electrophoresis. The electropherogram shows only one peak and no other peaks from minor components. This result shows that high purity HF-COLL-18/514cf was present in the final stage of purification.

Device:	P/ACE System 2000, Beckman Instruments GmbH, Munich, Germany
Capillary:	uncoated fused silica, 500 mm x 75 µm ID
Buffer:	100 mM sodium phosphate, pH 2.5
	0.02% hydroxypropylmethylcellulose
Temperature:	25°C
Injection:	20 s, corresponding to 120 nl
Run:	25 minutes
Current:	80 µA, constant
Detection:	200 nm

VII. Determination of the Molecular Mass of HF-COLL-18/514cf

Spectra could be obtained by means of MALDI TOF mass spectrometry on a Vestec BT II from the purified native HF-COLL-18/514cf from

fraction 25 in step V on two matrices (α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid). Peaks are found from the singly, doubly and triply protonated molecules. The molecular mass is determined to be 18507 u ± 20 u. Minor components cannot be seen.

For a more accurate determination of the molecular mass of the purified native HF-COLL-18/514cf, an additional mass spectrum was measured of fraction 25 of step V using an electrospray mass spectrometer (Sciex API III, Perkin Elmer, Langen, Germany). Peaks can be seen from the molecules with eight to eleven protonations. The average molecular mass of HF-COLL-18/514cf is determined to be 18494 u ± 3 u, the theoretical value being 18496 u (see VIII).

VIII. Determination of the aminoterminal amino acid sequence

By automated Edman sequencing with a Gas Phase Amino Acid Sequenator ABI 494 (Applied Biosystems, Perkin Elmer, Weiterstadt, Germany), the first 60 amino acids were determined. At the 21st position (Xxx), no amino acid was detected, as expected with cystein.

Val-Ala-Leu-Asn-Ser-Pro-Leu-Ser-Gly-Gly-Met-Arg-Gly-Ile-Arg-Gly-Ala-Asp-Phe-Gln-Xxx-Phe-Gln-Gln-Ala-Arg-Ala-Val-Gly-Leu-Ala-Gly-Thr-Phe-Arg-Ala-Phe-Leu-Ser-Ser-Arg-Leu-Gln-Asp-Leu-Tyr-Ser-Ile-Val-Arg-Arg-Ala-Asp-Arg-Ala-Ala-Val-Pro-Ile-Val

Thus, it has been established that the fragment is derived from collagen alpha 1 (XVIII), this protein having been known to date only on the cDNA level (Oh et al., 1994, Genomics, Vol. 19, page 494). The fragment starts at position 514 of the protein precursor, and the molecular mass shows that it ends at the last position but one of the precursor with the amino acid serine, i.e., is truncated by one lysine at the C terminus.

Example 2: Study of the Biological Effectiveness of HF-COLL-18/514cf

By the method illustrated in Example 1, a larger amount of material of more than 0.1 mg of HF-COLL-18/514cf was isolated from human hemofiltrate. The highly pure HF-COLL-18/514cf was employed in endothelial cell proliferation assays for the determination of its biological function. For this assay, bovine capillary endothelial cells from the adrenal cortex of freshly slaughtered calves were cultured as described in the literature (Folkman et al., 1979, Proc. Natl. Acad. Sci. Vol. 76, page 5217).

The proliferation assay was performed as described in the literature (O'Reilly et al., 1997, Cell, Vol. 88, page 277). Thus, the bovine capillary endothelial cells were washed with PBS (phosphate buffered saline, pH 7.4) and suspended in 0.05% trypsine solution. A cell suspension with 25,000 cells per ml in DMEM medium containing 10% FCS (fetal calf serum) and 1% GPS (glutamine-penicillin-streptomycin) was incubated in gelatine-coated 24-well plates (0.5 ml per well) at 37°C and 10% CO_2.

After 24 h, the medium was replaced by 0.5 ml of DMEM medium containing 5% FCS and 1% GPS and varying concentrations (from 0 to 1000 ng/ml final concentration) of the isolated high purity HF-COLL-18/514cf. After another 30 minutes of incubation, bFGF (basic fibroblast growth factor) was added to the mixtures to a final concentration of 1 ng/ml. After 72 h, the cell count in the mixtures was determined by crystal violet staining of the cells amd measuring the absorption at 600 nm. The HF-COLL-18/514cf added to the bovine capillary endothelial cells inhibited the bFGF stimulated proliferation of those cells in a concentration-dependent way. Half-maximum inhibition of the proliferation in this assay was reached with a concentration of 200 ng/ml HF-COLL-18/514cf.

In order to examine the specificity of the activity spectrum of HF-COLL-18/514cf and thus other possible biological functions thereof, proliferation assays were performed with non-endothelial cells. In tests with fibroblast cell lines, namely NIH 3T3 cells and LMTK cells, HF-COLL-18/514cf showed no significant effect and thus no antiproliferative activity, either.